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Cyclodextrins in Drug Delivery: Applications in Gene and Combination Therapy

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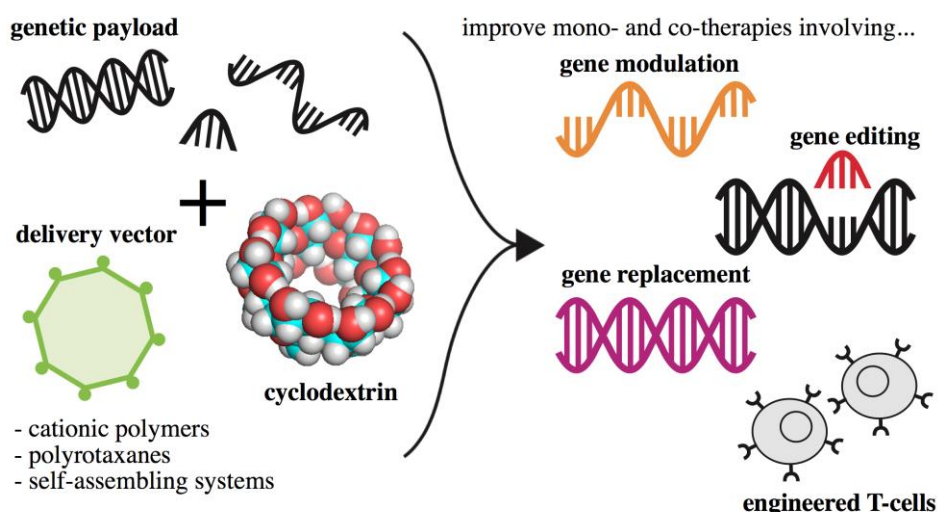
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1) ABSTRACT

Gene therapy is a powerful tool against genetic disorders and cancer, targeting the source of the disease rather than just treating the symptoms. While much of the initial success of gene delivery relied on viral vectors, non-viral vectors are emerging as promising gene delivery systems for efficacious treatment with decreased toxicity concerns. However, the delivery of genetic material is still challenging, and there is a need for vectors which show improved targeting, further reduced toxicity, and controlled release. In this article, we highlight current work in gene therapy which utilizes the cyclic oligosaccharide molecule cyclodextrin (CD). With a number of unique abilities, such as hosting small molecule drugs, acting as a linker or modular component, reducing immunogenicity, and disrupting membranes, CD is a valuable constituent in many delivery systems. Further, it shows great promise in combination therapies due to the ease of assembling macromolecular structures and wide variety of chemical derivatives, which allow for customizable delivery systems and simultaneous co-delivery of therapeutics. The use of combination and personalized therapies can result in improved patient health – modular systems, such as those which incorporate CD, are more conducive to these therapy types.



Graphical Abstract

2) INTRODUCTION

2.1 Gene Therapy

Table 1. FDA-Approved Gene Therapy Products

| Tradename | Manufacturer | Proper Name | Function | Status | |
|-----------|------------------------------|----------------------------|---|--------------|------|
| Imlygic | Amgen Inc. | talimogene laherparepvec | Genetically modified HSV immunostimulatory injected into melanoma tumors | FDA Approved | 2015 |
| Kymriah | Novartis Pharmaceuticals Co. | tisagenlecleucel | T cells are genetically engineered to express a chimeric cell surface receptor to target cancer cells | FDA Approved | 2017 |
| Luxturna | Spark Therapeutics Inc. | voretigene neparvovec-rzyl | AAV vector-based gene therapy for patients with inherited retinal disease and | FDA Approved | 2017 |

| | | | RPE65 mutations | | |
|-----------|------------------|-------------------------------|---|--------------|------|
| Yescarta | Kite Pharma Inc. | axicabtagene ciloleucel | CAR T therapy for adults living with certain types of non-Hodgkin lymphoma. | FDA Approved | 2017 |
| Zolgensma | AveXis, Inc | onasemnogene abeparvovec-xioi | AAV delivery of SMN1 gene for treatment of spinal muscular atrophy | FDA Approved | 2019 |

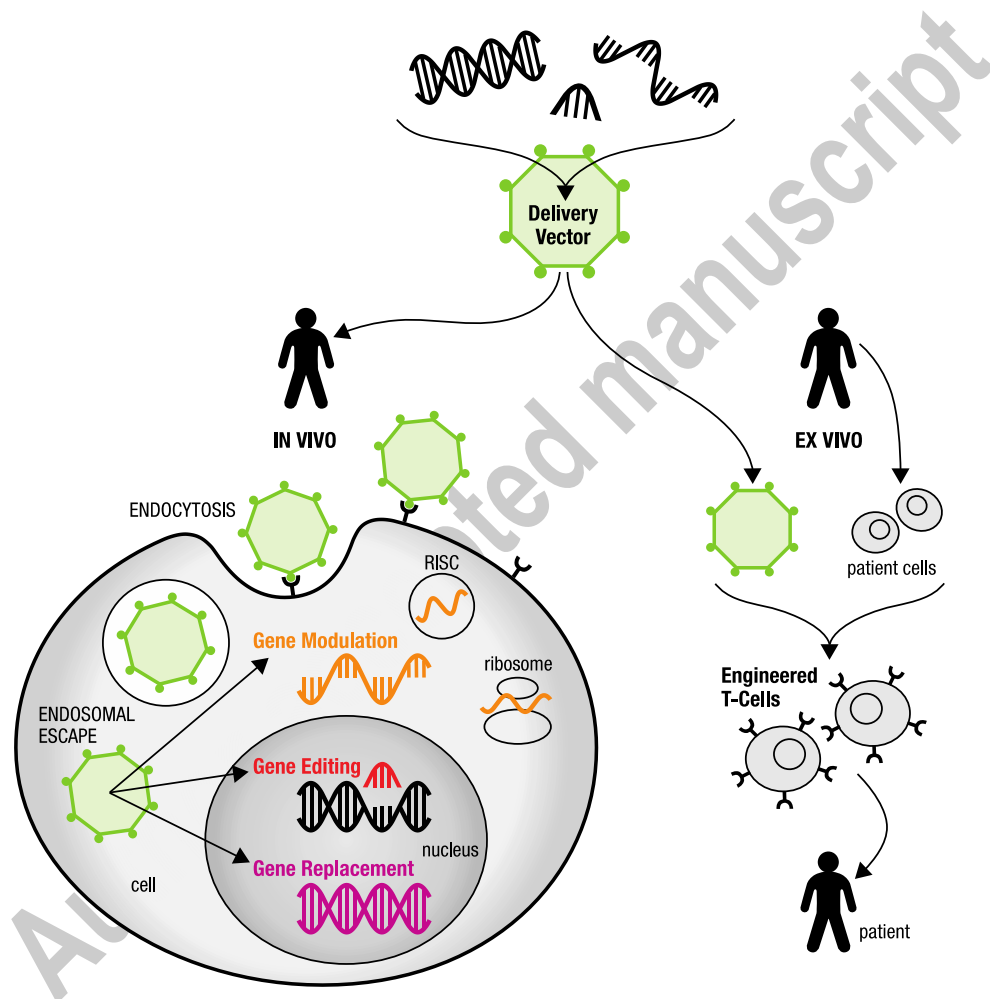


Figure 1. A schematic illustrating multiple types of gene-based therapy: gene modulation, gene replacement, gene editing, and engineered T-cell therapy.

The approved therapies listed in **Table 1** fall under the FDA’s “cellular and gene therapy products” category. Gene-based therapies recognized as such by the FDA can be organized into three main groups: gene replacement therapy, gene editing, and genetically engineered T-cell therapies. A fourth category, gene modulation through the use of RNA interference (RNAi), is categorized separately by the FDA, but is included in this work. (**Figure 1**) Gene replacement

therapy provides the patient's cells with a new copy of a missing or malfunctioning gene. This area has been studied since the 1970s [1], and the first gene replacement therapy, Luxturna [2], was approved by the FDA in 2017. (**Table 1**) Gene editing inserts, removes, changes, or replaces a patient's existing genetic code. The most recent and precise gene editing technique is clustered regularly interspaced short palindromic repeats (CRISPR), which is currently in clinical trials (NCT03399448, among others) [3]. However, there are no FDA-approved gene editing treatments at this time, and the CRISPR platform is used primarily to produce gene edited models to help understand mechanisms and develop other therapies [4, 5].

In addition to gene replacement therapy and gene editing, there are also genetically engineered cell-based therapies, such as chimeric antigen receptor (CAR) T-cell therapy, which consist of *ex vivo* genetic engineering of patient's immune cells so that they express CAR, enabling them to target and kill cancer cells [6–8]. There are multiple FDA-approved CAR-T therapies, the first of which is a platform called Kymriah, developed by researchers at the University of Pennsylvania [9] and approved in August 2017. However, even with a growing number of gene-based therapies entering the clinical space, the delivery of genetic material across biological barriers remains a significant challenge.

One effective means of delivering genetic material is through the use of viral vectors, which have evolved for such purposes [10]. Many viruses have been engineered to achieve therapeutic gene delivery, such as the herpes simplex virus (HSV), retro- or lenti-virus, adenovirus, and adeno-associated virus (AAV). These are very efficient methods of genetic delivery, but there are physiological barriers and safety concerns which limit their clinical implementation. Viral vectors have high immunogenicity, high cost, and can be inhibited by some drugs such as anticoagulants [11]. They also have package size limitations, and can only deliver genetic material under a certain number of base pairs (7.5kb for adenovirus, 4.5kb for AAV) [12].

To overcome these challenges, non-viral vectors are being designed as alternative delivery vehicles, with the long-term goal of achieving similar transfection efficiency to existing viral vectors while mitigating safety and cost concerns. This is achieved via the engineering of synthetic materials, such as lipid and polymer delivery vehicles, which use similar cell entry mechanisms to viruses but with improved safety, immunogenicity, and manufacturing [13].

Non-viral vectors have demonstrated several benefits for gene delivery, including better biocompatibility, large payload capacity, and the ability to specifically engineer their surfaces via functionalization [14, 15]. The condensation of genetic material using non-viral vectors also reduces stimulation of the innate immune response [16]. Although there are no FDA-approved non-viral gene therapies under the “cellular and gene therapy products” umbrella, there are quite a few systems which have been developed for RNA interference (RNAi) delivery [17], included here as a fourth category of gene modulation. One such system, Onpattro (patisiran, for treatment of polyneuropathy caused by hereditary ATTR amyloidosis; Alnylam Pharmaceuticals), is a lipid nanoparticle vector for RNAi delivery which was designated an orphan drug and approved by the FDA in August 2018. Patisiran is also currently in ongoing late-stage clinical trials (NCT03862807, NCT03997383) [18, 19]. Another is the spherical nucleic acid constructs being developed by Exicure [20], also currently in clinical trials (NCT03086278, NCT03684785) [21]. Multiple types of nucleic acids can decorate this nanoparticle system, including mRNA or siRNA modalities.

Non-viral vectors protect nucleic acids from nucleases and serum components [22], but often struggle to achieve delivery of significant genetic material. This can be caused by short

retention time or low therapeutic efficacy in targeted areas [11]. Some non-viral vectors still exhibit toxicity or immunogenicity, and can have issues with reproducibility. To fully transition to non-viral gene delivery vectors, improvements need to be made by exploring new non-viral delivery platforms and integrating new components into existing delivery vehicles.

2.2 Cyclodextrins

Cyclodextrins (CDs) have been used for years by the pharmaceutical industry to overcome drug delivery barriers such as solubility, bioavailability, and stability [23]. CDs are a family of cyclic oligosaccharides comprised of 6, 7, or 8 linked glucose units, called α -, β - and γ -cyclodextrin respectively [24]. They are used in a variety of applications, such as foods, cosmetics, air fresheners, and pharmaceuticals [24], and there are over 50 FDA-approved formulations in which CDs are used. Their wide usage is primarily due to their ability to form molecular inclusion complexes with guest molecules [25]. The three different types of CDs can form complexes with different binding coefficients and residence times [25]. These inclusion complexes can be used to stabilize or solubilize the guest molecules, and the host/guest interactions can be 1:1 or have a great excess of CDs – these interaction properties are based on the size and charge of the guest molecule in question [26]. Finally, CDs are also attractive because of their low toxicity and immunogenicity [27].

There are also many derivatives of CD which have been developed to improve the properties of natural CDs, and work around some of their limitations. The many hydroxyl groups found in CD can be used as reactive sites and replaced with alternate functional groups [28]. (Figure 2) These derivatives are commonly used in pharmaceuticals and can have improved properties such as solubility or membrane penetration [29]. CDs also have two different faces – the primary, narrower, face having primary hydroxyl groups, while the secondary, wider, face has secondary hydroxyls. The symmetry and chemical differences of these faces allow for compatibility with multiple modification types [30].

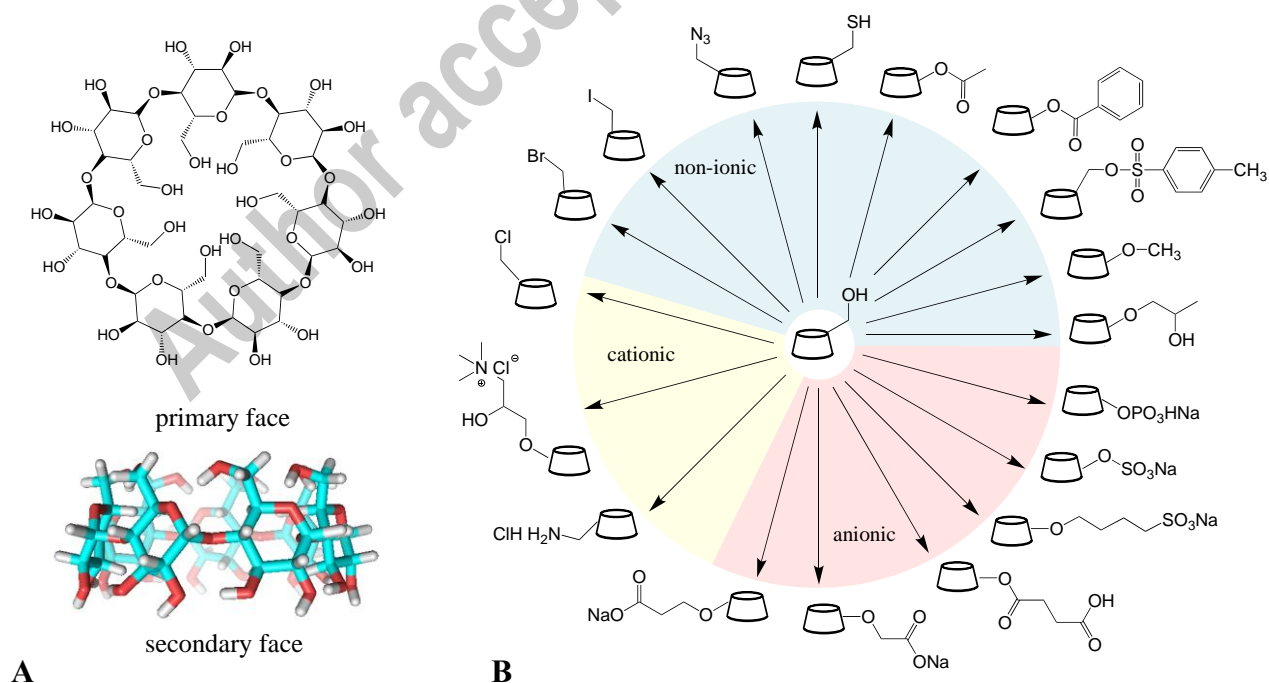


Figure 2. (A) The molecular structure of cyclodextrin (top) and a 3D conformer showing the primary and secondary faces (bottom). This structure is simplified to a truncated cone in (B)

where some common and commercially available derivatives of cyclodextrin are shown, broken down into cationic, non-ionic, and anionic groups.

In addition to the formation of derivatives, the hydroxyl groups can also be used for bio-conjugation or polymerization. Cyclodextrin has been polymerized using multiple crosslinkers, such as epichlorohydrin [31], ethylene glycol diglycidyl ether [32], isocyanate [33], and polycarboxylic acid [34]. These CD polymers show drug loading and controlled release using host/guest interactions. Other molecular edits can be done on only the head or base ring of the CD, creating different properties on each side. All of these options make the CD ring very modular, an attractive property for a delivery vector.

Based on these properties, many drug delivery systems involving CDs have been developed. These span from simple formulations, already approved for FDA use, to highly complex systems which can be engineered to specifically target disease states [35, 36]. The engineer-ability and modularity of CDs make them useful for the delivery of genetic material, among other drug cargo types. Resultingly, there has been significant work in the field of CDs as delivery systems, and there are a number of excellent existing reviews on the topic [11, 27, 30, 36–39]. In this work, we focus on the usage of CDs in gene and combination delivery applications, as well as attempt to comment on the trajectory of the field, based on current trends and existing literature reviews.

3) CYCLODEXTRINS IN GENE THERAPY APPLICATIONS

Cyclodextrins have been extensively researched for multiple types of gene monotherapy. They have been used in systems to improve gene editing, replacement, and modulation within cells through the delivery of DNAs and RNAs. While there do not appear to be any current applications in CD-based *ex vivo* T-cell engineering, this may well change if the technology successfully moves *in vivo* and transient expression of CAR antigens (through mRNA delivery) becomes more common [40].

Although “pure” CD vectors for gene therapy exist, CDs are more typically one of multiple components in gene delivery systems. The addition of CD can impart many of its positive properties to the system as a whole, such as hosting small molecule drugs, acting as a linker or modular component, reducing immunogenicity, and disrupting membranes. In some cases, CD directly interacts with the genetic cargo. In others, genetic cargo is held by a different system component, but CD still plays an integral role such as vehicle stability or ensuring endosomal escape. Without these properties, gene delivery vectors cannot effectively deliver cargo to its desired destination. By imparting such capabilities to the larger system, the addition of CD allows for more successful delivery in gene therapy applications.

3.1 Cationic Polymers

Cationic polymers are widely utilized in gene delivery due to their positive surface charge, and are usually made of cationic monomers containing amine groups [41]. This allows for electrostatic interaction between the polymer and any nucleic acids which have a negative charge, such as plasmid DNA (pDNA), microRNA (mRNA), small interfering RNA (siRNA), messenger RNA (mRNA) and even cas9 RNP or S1plex [42]. This interaction causes a condensation of polymer and nucleic acid into nanoparticles called polyplexes [43]. These polyplexes hold the genetic material while also protecting it during delivery.

In addition to the advantages noted above, cationic polymers are attractive non-viral delivery vectors because they are highly customizable, both in size and type of genetic material used for delivery – this stems in part from the fact that cationic polymers are very chemically diverse. They can also be produced to have long shelf lives, indicating potential for an easier path to clinical use [41]. Finally, cationic polymers are believed to exhibit the proton sponge effect, where protons and counter ions are drawn into the endosome by the presence of the polyplex. This causes an influx of water in an attempt to balance the osmotic gradient, which ultimately results in the bursting of the endosome, allowing the polyplex to escape and deliver its genetic cargo within the cell [43]. However, cationic polymers are currently limited in their use, in part due to low uptake and efficiency, toxicity, and immunogenicity. To help mitigate some of these concerns, many groups have worked to introduce CDs to traditional cationic polymer systems, and have shown that these CD-integrating formulations successfully deliver pDNA [44–49] and various RNAs [16, 50–52] for gene editing and gene modulation. In general, these delivery systems show greater efficacy and lower toxicity than similar formulations which do not incorporate CD.

Other groups are exploring CD mediated polycations, which utilize CD as the base of the system, and incorporate cationic charge by modifying CD's hydroxyl groups [27, 53–58]. CDs modified with cationic moieties can directly form polyplexes with nucleic acids, much like existing cationic polymer systems [53, 54, 58]. These electrostatic interactions allow for self-assembly into stable structures. When these CD polycations also exhibit amphiphilic properties, they can form nanostructures where nucleic acid cargo is compacted and encapsulated within [53, 59]. The complex which CD can form with host molecules enables shielding of the therapeutic cargo, up to macromolecular scale [59]. CD also naturally exhibits membrane disruption, leading to enhancement of permeability for its cargo [27]. This disruption is beyond that of the proton sponge effect alone, as it can improve delivery to the cytosol not only through enhanced uptake but through improved endosomal escape [60]. This capability is important to note as some studies suggest that particles which include CD and their derivatives may also be able to enter cells by the endocytotic pathway [61, 62].

The cationic polymer polyethyleneimine (PEI) has been used extensively as a non-viral gene delivery vector, and is an example of the inherent cytotoxicity of cationic polymers with strong charge density. While there are both linear and branched forms of PEI, the most effective gene delivery is achieved using the branched form, as it condenses DNA to a greater extent [12]. However, as the PEI polymer network becomes larger and more branched, it also becomes more cytotoxic [63]. Regardless, PEI is considered by many to be the 'gold standard' of non-viral gene delivery, due to its strong cationic charge and buffering capability, leading to high transfection efficiency. PEI, like other polycations, also exhibits the proton sponge effect, combining its strong polyplex formation with intrinsic endosomolytic activity [64].

Overall, the use of PEI is limited by its severe toxicity, and reducing dosage to a non-toxic level results in low transfection efficiency and short duration of gene expression [64]. To mitigate these issues, several alterations to and derivatives of PEI have been explored, including the usage of CDs [16, 65–67]. By incorporating CD into PEI delivery systems, toxicity concerns can be alleviated by shortening the molecular weight of PEI used while retaining its gene transfection properties. In fact, formulations which include CD show improved DNA condensation over PEI alone [48]. An example of CD incorporation is the synthesis of PEI- β -CD, through creation of amine-reactive CD and conjugation to the amines found in branched PEI

[68]. Even when only ~10% of the amines are conjugated with β -CD, the resulting polymer has significantly reduced cytotoxicity as compared to PEI alone [68].

Other recent studies have taken advantage of CD's modularity, exploiting host/guest interactions to self-assemble cationic polymers that are part of a larger macromolecular system [53, 69]. In one example, a cationic β -cyclodextrin-modified PEI was synthesized, in which the branched arms of PEI were modified to terminate with a β -CD [50]. This allowed for larger self-assembly, encapsulating the polyplex within a pH responsive layer, which serves as both an added protection layer for the genetic payload and as a method for programmed endosomal escape and delivery [50]. These modifications allow for more efficient gene delivery, which can additionally reduce toxicity indirectly by reducing the dosage required to reach therapeutic transfection levels. Another example is the creation of PEI chains which terminate in a β -CD molecule. This allows for utilization of the self-assembly characteristics of CD to create a protective coating for the resulting polyplex, attaching polymers such as poly(ethylene glycol) (PEG) as an outer shielding layer [45]. By incorporating PEG, this delivery system may benefit from reduced immunogenicity and enhanced circulation times – both properties which can indirectly improve delivery efficiency. Looking forward, complex gene editing technology and the delivery of CRISPR-associated protein-9 (Cas9) could be improved using similar delivery techniques. While direct protein delivery is possible, delivery of pDNA which codes for Cas9 and RNA is generally preferred as it is much easier to encapsulate and deliver intracellularly. PEI- β -CD shows promise as an effective vehicle for this approach [70], both *in vitro* and *in vivo* [71].

While PEI is very commonly used in conjunction with CD, there are several other cationic polymers which have similar limitations and can take advantage of the improvements that CD can provide. One example is poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) which [72], while less common, can exhibit up to 90% of PEI's delivery efficiency and contains only tertiary amines, which are less reactive than their primary counterparts [73]. Similarly to PEI, larger molecular weight PDMAEMA shows better transfection efficiency, but increased toxicity. However, incorporating CD as a core to hold PDMAEMA [74] has proved successful for gene delivery, improving efficiency and allowing for reduction in dosage and toxicity [49, 75]. The cationic lipid Dioleoyl-3-trimethylammonium propane (DOTAP) has also been formulated with the addition of CD. In this case, a CD derivative was used (carboxymethyl- β -cyclodextrin), which exhibits more lipid-like (amphiphilic) properties better suited to liposome formation. When CD was introduced, increased transfection occurred in certain cell lines [76].

There are also naturally non-cationic polymers used in gene therapy which can be functionalized with polycations to provide DNA condensation properties. Poly(glycidyl methacrylate) (PGEA) can be functionalized with ethanolamine or ethylenediamine to produce a gene delivery vector [77, 78], and when combined with CD, the polymer can self-assemble on various surfaces, such as gold or iron nanoparticles [79]. This allows for the introduction of gene delivery to many existing delivery systems – gold nanoparticles especially have been studied extensively and their interactions within the body are well characterized. Similarly, linking ethanolamine functionalized PGEA to a β -CD backbone showed improved uptake efficiency, pDNA transfection efficiency, and siRNA silencing efficiency as compared to the 'gold standard' 25 kDa PEI [80]. This system also took advantage of the self-assembling properties of CDs, and used the host/guest complex to create a PEG-coated and folate-targeted outer shell for additional targeting and stealth.

Overall, the incorporation of CDs into cationic polymer delivery vectors serves to improve transfection efficiency and lower cytotoxicity while also introducing modularity. Many modifications can be achieved building from CD's molecular structure and taking advantage of self-assembly. This in turn allows for simple improvements to nanoparticle design to optimize delivery efficacy. CDs can also be modified directly to exhibit cationic and polyplex forming capabilities comparable to cationic polymers.

3.2 Polyrotaxanes

Polyrotaxanes are often cationic polymers themselves, but have additional characteristics which make them unique. A linear polymer backbone, such as PEG, interacts with CDs such that they thread along the PEG axis, forming a supramolecular structure [81, 82]. In a polypseudorotaxane, CDs can freely slide along and off the linear backbone. Polyrotaxanes have the ends of the backbone capped with a bulky blocking group, so that CDs are trapped in the structure until release is triggered [11], typically by changes in pH or redox state [50] to target areas such as tumor sites. Although they are trapped by the blocking group, CDs are free to slide along the length and rotate around the central polymer backbone [83].

Once threaded onto the backbone, CDs are often modified with various conjugates, some of which are cationic and allow for the direct condensation of genetic material such as pDNA and RNAi [78]. The major advantages of polyrotaxanes include their reactive release of CDs and cargo, and their biomimicry. Chemically, they appear to mimic histones. Morphologically, they have been compared to the nucleosome [46]. Together, these advantages seem to allow for innate macrophage targeting, which can be useful in immune applications. Threaded alpha CDs along a PEG central backbone created a polyrotaxane which was shown to be uptaken by macrophages via the macrophage mannose receptor (MMR) [83]. In fact, it appears that macrophages can even be selectively targeted in this fashion, delivering payloads specifically to macrophages exhibiting the M2 phenotype. In nanoparticle systems, CD has been found to form host/guest inclusion complexes with R848, a TLR7 and TLR8 agonist that drives the M1 phenotype in macrophages [84]. With the addition of macrophage targeting, such systems could prove useful for gene delivery.

The cationic conjugates added to CD are often large polymers themselves. PGEA [78] and PEI [46] can be functionalized to CDs and threaded along the central polymer backbone. This results in a larger architecture which is similar to that of a large molecular weight branched polycation, but without the associated toxicity [46]. Large cationic polymers can also be integrated to the polyrotaxane system by acting as the central backbone. By threading CD onto cationic methoxy-poly(ethylene glycol)-*b*-poly(ϵ -caprolactone)-*b*-poly(ethylene imine) (MPEG-PCL-PEI), a complicated copolymer with the ability to form polyplexes with DNA was formed [85]. As a block copolymer, one section of the system holds the CDs while another is primarily responsible for holding genetic material. This allowed for hydrogel formation with sustained gene release.

As with the more general cationic polymers, the introduction of CD to polyrotaxanes allows for improved transfection and reduced cytotoxicity, with the added advantages of triggered release dependent on the bulky end group, and the potential for macrophage targeting.

3.3 CD for Supramolecular Assembly

CDs can be crosslinked to each other to form large polymer networks, or used as crosslinkers themselves to create supramolecular structures [46, 75, 86]. As mentioned

previously, the hydroxyl groups found on CD are reactive, and can be modified in several ways to account for chemistries which require alternate functional groups. (**Figure 2**) Reversible, but stable, host/guest interactions with CD can serve as structural crosslinkers as well. Crosslinked systems are often advantageous for delivery. Larger particles are more likely to extravasate from the bloodstream, and variations in crosslinking can affect stiffness of the polymer matrix – both examples of properties which may allow delivery systems to overcome biological barriers and improve accumulation and delivery at the target site.

By crosslinking small cationic polymers into larger cationic networks, the advantages of large molecular weights can be obtained while decreasing associated toxicity [47, 87]. *In situ* crosslinking can allow for injectable delivery vehicles, allowing for larger gene delivery depots or localized delivery without requiring invasive implantation [85]. Crosslinking via CD host/guest interactions can allow for shear-thinning behavior during injection, as well as rapid gel healing after removal of shear, allowing for injection directly into sensitive tissue [88]. These approaches may be useful in patient cases where local gene delivery is preferred, such as when the disease state only occurs in a specific location [89, 90]. Due to the novelty of gene editing technology, there are still concerns about off-target effects, and localizing therapeutics in this fashion helps to mitigate these concerns. Collectively, crosslinking of non-viral polymeric delivery systems can reduce both toxicity and off-target effects.

PEI has been crosslinked using CD in multiple fashions and by several groups [45, 91–93]. By activating the hydroxyl groups on the outside of β -CD or γ -CD (using reagents such as 1,1-carbonyldiimidazole), crosslinking can be achieved with the amino groups found on PEI [91]. Low molecular weight PEI can also be crosslinked via disulfide bonds with cyclodextrin [47]. Both crosslinking methods show lower cytotoxicity and higher transfection efficiency for the delivery of pDNA compared with those of a PEI 25 kDa control. Polycaprolactone (PCL) is another polymer which has been chemically linked to CD, to form larger networks such as amphiphilic star polymers [75]. In this system, PCL arms were linked to the hydroxyl groups on a single β -CD, creating a star polymer with 21 arms. CD serves as the core of the particle, and acts as a multifunctional initiator [75]. These crosslinked polymer systems are just as stable as their individual polymer parts, but allow for greater control over individual polymer chains. Although these examples are single polymer systems, crosslinking in this fashion would also allow for the production of co-polymer systems – which may hold interest in future combination applications.

Crosslinking via CD hydroxyl groups is very effective, but can also require harsh chemicals or temperatures depending on the desired chemistries. An attractive alternative is to exploit the innate ability of CDs to self-assemble utilizing host/guest complexes [94, 95]. Adamantane (Ad) is a non-functionalized hydrocarbon which has a number of interesting derivatives. Cyclodextrin forms an inclusion complex with Ad which has a high association equilibrium constant, on the scale of 10^4 – 10^5 M^{-1} ($\log K=5.04$) [25, 96]. CD and Ad self-assemble under physiologic conditions to create reversible but stable structures [97]. Their usage in macromolecular design allows for assembly without complicated synthesis and separation steps [48]. The host/guest complexation does not produce any heat or byproducts, and the individual components have been shown to be non-immunogenic and non-toxic [27, 98]. The utilization of the CD-Ad complex allows for construction of delivery vehicles in a stepwise fashion, taking advantage of the noncovalent hydrophobic complex to form multiple self-assembling structures such as nanoparticles and hydrogels [95, 99]. The stepwise nature of these interactions also lends itself to the engineering of systems which are layered or blocked co-

polymers, further increasing modularity. A number of nanoparticles fabricated using CD-Ad interactions have been reported, and these particles successfully deliver genetic material to various cell types and *in vitro* models [50, 95, 100, 101].

Any material type can take advantage of the host/guest interactions of CD and Ad, even inorganic materials, such as gold and iron nanoparticles [79]. Often, these structures utilize cationic polymers in their systems, such as poly(*N,N'*-bis(acryloyl)cystamine-poly-(aminoalkyl)) (PBAP) polyplexes, which were crosslinked using CD/Ad interactions [42]. These polyplexes showed similar or better transfection efficiency to Lipofectamine 2000 (commercially available agent) with no cytotoxicity, as compared to the severe toxicity associated with Lipofectamine 2000. PEI is also commonly modified using the CD/Ad assembly to enable PEG shielding [45], cell targeting [100, 101], or even crosslinking to other PEI strands by modification of PEI with an adamantyl end functional group [48]. Multiple guest molecules can be modified with adamantyl functional groups in this fashion to take advantage of this natural self-assembly. (**Figure 3**) These guest molecules can function to complex with genetic cargo or introduce targeting to the delivery system, and are easily modified to fit the system's needs. An adamantane-ended linear poly(poly(ethylene glycol)ethyl ether methacrylate) polymer was formed to allow for self-assembly of gene delivering star polycations [102]. CD/Ad has also been used to link multiple star polymers to each other, allowing for pDNA delivery [95].

Benzimidazole can be used as a guest molecule in a similar fashion to Ad, creating a host/guest interaction that is pH mediated and allows for triggered decomposition within the lysosome [51]. Other groups have used the benzimidazole/ β -CD complex for chemotherapeutic delivery [103]. Ferrocene (Fc) has also been used in host/guest fashion. With a strong initial association, ($\log K=3.68$) [25] oxidation of Fc leads to dissociation of the complex, creating a redox-responsive system for delivery [82]. Regardless of the molecules used to form the CD complex, all these modifications impart additional properties to existing gene delivery systems. Modification with functional groups can be used to attach many molecule types, from large polymer networks to small targeting moieties.

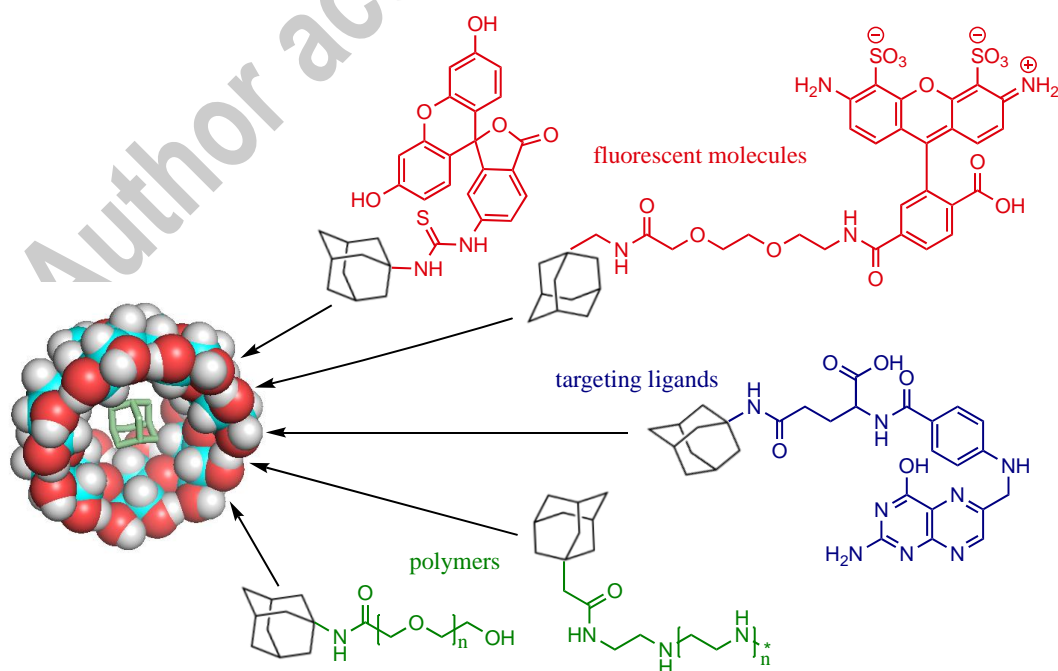


Figure 3. Adamantane and Adamantyl functionalized molecules form host/guest inclusion complexes with cyclodextrin, allowing for self-assembly. 3D conformer of CD/Ad complex (left) and example molecule types which take advantage of this system (right) are shown: fluorescent molecules FITC and Alexa Fluor 488, targeting ligand folic acid, and polymers PEI and PEG (clockwise).

The addition of cell targeting creates a huge opportunity for personalization and modification of CD-modified gene delivery vectors. The addition of PEG or folate to the surface of delivery vectors can both improve stability and reduce immunogenicity through steric hinderance and immune cell evasion. Folate specifically also allows for cell targeting, as it is overexpressed on many human cancers and can trigger cellular uptake via endocytosis [57, 80]. More specific targeting can be integrated as well via the addition of tumor-targeting peptides such as arginylglycylaspartic acid (RGD) [50]. Using CD-Ad interactions to add RGD targeting to PEI nanoparticles allows for cell-specific uptake [100, 101].

The modulation of surface properties also allows for combination modifications. Multiple different types of Ad-modified molecules can be obtained: two targeting ligands (Ad-PEG-FA and Ad-PEG-LA), a cationic polymer (Ad-PEI), and a fluorescence agent (Ad-FITC) are such examples [104]. By combining these Ad-modified molecules, a single system which hosts diverse functions can be obtained and used to achieve efficient eradication of tumors [104]. Self-assembly is simple and can occur in ambient or physiological conditions with no toxic byproducts, and the integration of modularity allows for stimuli responsive, targeted gene delivery.

3.4 Pure CD Vectors

Although less commonly used in gene delivery, there are other ‘pure’ CD vectors which utilize CD’s properties to provide gene delivery. These vectors use CD as the main constituent of the gene delivery system, as compared to the previously explored works which integrate CD to perform a secondary role. Examples include cyclodextrin-based nanosponges [105], nanoparticles [84, 106, 107], vesicles [108], and hydrogels [11]. Typically, these formulations are composed of CD derivatives. Native CD does not have the innate ability to complex strongly with genetic material, and therefore has limited transfection ability. However, systems have been successfully engineered which allow for DNA condensation within the CD pocket [109]. Gene delivery can be achieved through the usage of carboxymethyl- β -cyclodextrin, a simple modification which is commercially available [110]. Some groups have had success delivering DNazymes, which form 1:1 polyplexes with CD and can target and suppress genes of interest [107, 111]. Other groups have utilized the host/guest ability of CD to create responsive drug delivery systems, which function through binding competition or more effective isomerization, where the weaker complex CD forms with genetic material is an advantage, as it can be undone by a molecule with a stronger affinity [112].

Cationic-modified and amphiphilic CDs are also used often in gene delivery systems. Cationic functional groups and hydrophobic anchors can be added to one or both faces, creating medusa-like, skirt-shaped, or bouquet-like CDs by adding polymer chains to the primary, secondary, or both faces respectively [27, 37]. (**Figure 4**) These amphiphilic CDs then bind to genetic material and self-assemble to form nanostructures [57, 69, 113, 114]. These structures vary in shape, and can be liposomal, micellar, or random in nature. Cationic-CD/genetic material complexes have been shown to deliver siRNA effectively *in vitro* and *in vivo* with favorable

toxicity profiles, successfully silencing the mutant protein in Huntington's disease [55, 115]. pDNA has also been successfully delivered by cationic-modified amphiphilic CDs [53, 54, 116]. Adding cationic components to CD seems to be the most direct method to complex genetic material. However, some groups prefer to create polyanionic CDs instead, which can interact with traditional cationic polymer and DNA polyplexes. Sulfobutylether- β -cyclodextrin is one such polymer [117].

Other examples of functional groups being added to CD include the addition of cationic primary amines [106] and 2-hydroxypropyl [118]. 2-hydroxypropyl- β -cyclodextrin (HP- β -CD) is the most widely used modified CD, and is typically implemented to improve the aqueous solubility of lipophilic drugs [119]. This occurs due to the amphiphilic properties imparted by the additional polarity of the modification. It has been shown to be well tolerated in humans [120]. This CD derivative can also be further modified via click chemistry to create a wide variety of functionalized CDs, each with different properties. These 'pure' CD systems have a variety of advantages. They can self-assemble into a number of nanoshapes including micelles, spheres, and vesicles [109]. They can be easily modified to add targeting groups, allowing for targeted gene delivery, and can be reacted in a number of ways to create infinite combinations of properties at the primary and secondary faces.

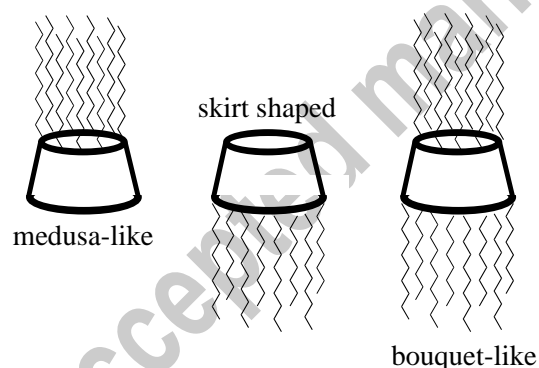


Figure 4. Amphiphilic cyclodextrins can be formed by adding polymer chains to the primary and/or secondary faces of CD.

4) CD IN COMBINATION THERAPY: CURRENT AND FUTURE DIRECTIONS

Combination therapy uses two or more therapeutic agents, and has been shown to be an effective treatment strategy in oncology. Gene, immuno-, and chemotherapies have been used in various combinations to improve patient outcome [121–123]. While combination therapy is widespread in cancer treatment, it also holds promise for treating other complicated disease states such as Alzheimer's [124], cystic fibrosis [125], and infections [126]. The usage of multiple therapeutics simultaneously comes with a number of positive effects. Combination therapies allow for use of reduced individual dosages, which allows for therapeutic effect without hitting dose-limiting toxicity levels. The use of multiple drug types can also result in synergistic effects, where the outcome is better than the sum of the individual drug improvements [51]. Combination therapy also helps to reduce the likelihood of acquired resistance, by introducing alternate mechanisms of action [127]. In cancer especially, acquired resistance remains a large obstacle to many otherwise efficacious therapeutics.

Due to the number of positive attributes combination therapy offers, it is a current and promising field of study. There are thousands of clinical studies investigating combination

therapies, which results from the fact that the possible drug combinations are seemingly endless. In the current climate of clinical treatment, which is moving away from a “one-size-fits-all” approach and towards the more widespread implementation of precision medicine, combination therapy is uniquely qualified to overcome limitations of single therapies by introducing secondary agents. Gene delivery is currently limited by an inability to deliver enough cargo within the cell, due to the many biological barriers that delivery systems face. Secondary therapeutics can clear the way for gene delivery, by down-regulating the immune cells that capture circulating delivery systems, or beginning to break up the tumor microenvironment to improve distribution within solid tumors, for example.

However, in order to effectively deliver multiple therapeutic cargo types, delivery systems must be specifically tailored to optimize for combination therapy. Therapeutics typically have alternate mechanisms of action and resultingly, different final destinations within the body. The usage of CD is advantageous in these cases due to its ability to fulfill multiple functions: hosting small molecule drugs, acting as a linker or modular component, reducing immunogenicity, and disrupting membranes. CD forms inclusion complexes with many different drug types, including chemotherapeutics [59, 104, 128–130], immunomodulators [105, 118, 131], cytokines [132], and even proteins [82, 133]. When the modularity of CD hydroxyl groups is used to create gene delivery systems, the CD pocket often goes unused, and could be easily loaded with a small-molecule drug to integrate a secondary therapy type such as chemo- or immunotherapy. Similarly, delivery vehicles which use the CD pocket can often be modified at either of the CD faces to add an additional delivery component to the system. This concept makes it fairly easy to create CD delivery systems which can hold two or more payloads with variable properties and applications.

4.1 Existing CD delivery systems for Combination Therapy

CD delivery systems for combination therapy are generally very similar to those used in gene mono-therapy and covered in the previous sections of this work. While genetic material is delivered via cationic polymers, supramolecular assemblies, or pure CD vectors, alternate therapies are loaded into secondary parts of the system [65, 130]. These therapies are commonly chemotherapeutics or immunotherapies, and these complex systems can allow for the incorporation of tracking agents, active targeting, and responsive properties, among others. The ability to choose from multiple methods of therapy incorporation allows for modularity and the ability to personalize a drug delivery system, both advantages which should allow systems to be engineered to produce the best therapeutic results.

Chemotherapeutic drugs are one of the most commonly administered cancer treatments, and there are over a hundred used clinically in both mono- and co-therapies. Due to their promiscuous toxicity, effective delivery of chemotherapeutics aims to maximize exposure to tumor cells while minimizing exposure to healthy tissues. While these drugs are heterogeneous, they are typically small molecules with limited water solubility. Resultingly, they often have high affinities to complex with CDs, and can easily co-loaded when the CD pocket is not in use for gene delivery applications. Doxorubicin (DOX) is a very common chemotherapeutic which has been co-delivered via host/guest interactions with CD [51, 59, 60, 128, 134]. As an example, polycationic brushes have been used as multifunctional carriers, delivering both the p53 gene (functions as a tumor suppressor), held by the cationic comb block, and DOX held in the CD pocket via host/guest interactions [59, 134]. Another gene co-delivery system used PEI and CD for the loading of nucleic acids and DOX respectively [51, 128]. DOX interacts directly with

DNA, breaking it down to cause cell death. As it has the same final destination (intracellular, nucleus) as genetic cargo, the addition of DOX to a gene delivery system is fairly simple and typically does not require much additional optimization. However, for nucleic acids which illicit effects within the cytosol (RNAi), additional design considerations may improve intracellular trafficking to the nucleus.

Paclitaxel (PTX) is another small molecule chemotherapeutic which can undergo host/guest interactions with CD, and has been successfully co-delivered with genetic cargo [117]. Researchers have seen successful co-delivery of the B-cell lymphoma-2 conversion gene Nur77 and PTX using an injectable hydrogel system, which incorporated PEI, PCL, and CD [85, 129]. Using this system, PTX delivery was measured over the course of a week. This time scale is important to note as PTX targets microtubules, disrupting mitosis. If PTX is not administered during a mitotic cycle, effects are limited. Although it is not a traditional chemotherapeutic, there has also been success with the co-delivery of genetic material and TPP, a photosensitizer which creates reactive oxygen species [135]. When complexed with CD as a guest molecule, TPP can impart additional tumor-killing effects as well as improved endosomal escape.

Co-delivery of these small molecules is not limited to the utilization of host/guest interactions alone. There are other CD systems in which DOX is encapsulated within the complex. In one such study, DOX was encapsulated in the center of a nanoparticle decorated with targeting ligands, PEI, and a fluorescent agent [104]. Another nanoparticle system loaded DOX into a PAMAM dendrimer core [60]. In these applications, there is a reduced change of drug leakage, or delivery before final destination has been achieved. In the case of these toxicity chemotherapy drugs, more stable encapsulation may be preferred to reduce off-target effects.

In addition to chemotherapeutics, gene therapies are also often co-delivered with immunotherapies. Immunotherapeutics can be small molecules (immunomodulators, cytokines), proteins (antibodies, checkpoint inhibitors), or even cell-based therapies. Many of these molecule types have stability or solubility concerns, and are limited in their efficacy when administered in free drug form. Due to the wide range of sizes and properties of these therapies, immunotherapy delivery systems are varied. The modularity of CD, as well as its ability to complex with guest molecules of multiple types, show promise for use in immunotherapy applications. Additionally, delivery vectors formulated with CD have been shown to have high macrophage affinity [136], which could be advantageous for the introduction of immunotherapies.

Combination therapies also can incorporate tracking agents, active targeting, and responsive properties, further improving modularity. There are existing nanoparticle formulations for combination therapy which demonstrate this modular nature very well. CD coatings can be used to allow multiple molecules to dock to the surface via Ad/CD complexes. In one such system targeting ligands, PEI, and a fluorescent agent were attached to the surface via Ad/CD complexes [104]. Ad/CD complexes self-assemble, making the fabrication of this particle facile. This allowed for targeted delivery of genetic cargo, as well as tracking of the delivery system.

Although this review has focused mainly on non-viral vectors which incorporate CD, there are a few usages of CD in viral gene delivery as well, some of which are combinatorial. By linking CD to the surface of an adenovirus, reduced immunogenicity can be achieved [86], as well as increased modularity via delivery of a secondary therapeutic, such as TGF-beta inhibitor [65]. While these examples focus specifically on single delivery systems which hold multiple therapeutic cargo types, it is also important to note that there is a very large body of work in which co-delivery does not utilize a single delivery vector. In many of these applications,

delivery systems are used to improve efficacy of one therapeutic, and another is administered as free drug. In many cases, these studies serve to check the interactions of two drug types before time is spent optimizing a delivery system for both. However, in some cases, synergy of drugs may only be seen when delivery efficiency is above a certain level. To that point, there are a number of existing mono-therapies which could show significant improvement if coordinated delivery of multiple therapeutics could be attained.

4.2 Future Combination Therapy Applications

The previous examples were created with the intent of co-delivering multiple therapeutics, but there are other delivery vehicles which have been created for monotherapies and may be easily translatable for use in combination therapy. These typically fall into two categories: delivery systems which were co-administered with a free drug, and delivery systems which have a currently unused component.

In the first category, a pure beta-CD nanoparticle was fabricated to hold DNzyme, which did little to kill cancer cells (10-20% cell death) on its own, but was found to work synergistically with DOX to improve effects [107]. In this work, DOX was not loaded into the nanoparticle itself, but was instead administered in a soluble form which mimics systemic administration. However, DOX is known to form inclusion complexes with CD, and could likely be delivered more consistently and for longer periods of time if co-delivery was explored. Similarly, the immunotherapeutic NLG919 was found to form host/guest complexes with a CD derivative, improving solubilization and anti-tumor efficacy when delivered in concert with free PTX [137]. By co-delivering these two therapeutics, even greater efficacy may be possible. Another study showed that the delivery of R848 (toll-like receptor agonist) from a CD-based delivery system could promote the M1 macrophage phenotype and enhance the efficacy of the checkpoint inhibitor anti-PD-1 [84]. These, and other, immunotherapy applications could be improved through the usage of smart delivery system design for co-therapeutic delivery.

An example of a delivery system with an unused component is a co-delivering cationic nanocapsule [138]. A cationic poly(cyclodextrin) and alginate shell was formulated for the delivery of 4-hydroxy-tamoxifen, a selective estrogen receptor (ER) modulator used for the treatment of breast cancer. A small molecule drug, tamoxifen, was loaded into the nanocapsule via inclusion with the CD pocket. The cationic nature of the CD polymer in this system brings forward the idea of integrating gene delivery as well, through the formation of polyplexes. In this system specifically, the cationic charge is used to create layering, via interactions with the negatively charged alginate, but the idea still holds as inspiration for similar systems which already utilize cationic polymers. These are only a few examples of such systems, but the numerous methods which have been used to create delivery systems for chemo-, immune-, and gene therapy support the theory that as more complex systems are developed, it will become easier to incorporate multiple therapeutic types and optimize for both.

5. CONCLUSIONS

The utilization of CDs in gene delivery vectors can improve overall efficacy of the system. Formulations incorporating CD show more efficient gene condensation, delivery, and transfection, compared to the common standards tested: 25kDa PEI and Lipofectamine 2000. These improvements can come from direct action of the CDs, as is the case when improved membrane disruption occurs, or can be indirect applications – improvements to stability and size control can enhance passive targeting and uptake. Additionally, CDs have been shown to reduce

related toxicities. Many efficient gene delivery vectors currently come with significant side effect, and these can be mitigated through smart engineering of the delivery system – including the introduction of CD.

CDs are very biocompatible, and have already been approved for clinical applications. As of January 2020, 18 clinical trials in the United States with the term “cyclodextrin” in the study title are listed as completed (based on a ClinicalTrials.gov search). Another 9 trials are listed as either active or recruiting. This makes the path to market marginally easier, which is not often the case when adding components to a delivery system. There are several current studies which utilize CDs in gene delivery, and as more uses are discovered, the utility of the CD system increases. There are also a fair number of systems which have shown efficacy through *in vivo* testing, on both small animals and large non-human primates [139], further supporting the idea that this toolbox of gene delivery vectors, including those which incorporate CD, is growing. (Figure 5)

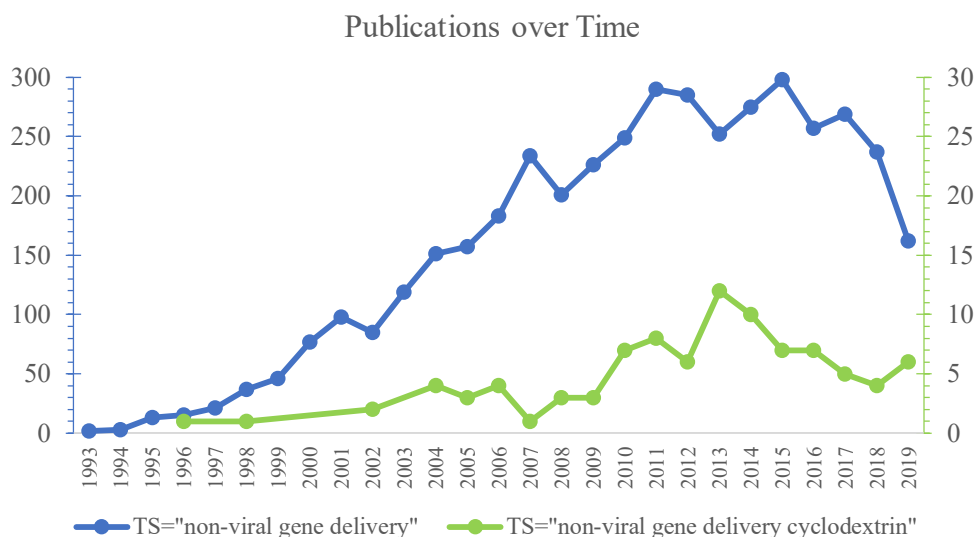


Figure 5. Publications on non-viral gene delivery over time. Search terms (on pubmed.gov) used were TOPIC = “non-viral gene delivery” or TOPIC = “non-viral gene delivery cyclodextrin”. Far fewer gene delivery papers are published on CD each year, but both searches seem to follow the same trend.

Over the past 10 years, the body of work which explores cyclodextrins for gene delivery has been growing with a fairly consistent number of new publications each year. The content of this work has also been consistent. CDs are still crosslinked using epichlorohydrin and isocyanate crosslinkers, and CDs and cationic polymers continue to be incorporated to balance polyplex stability and toxicity concerns. Adamantane remains the chemical of choice for many CD-based self-assembling nanoparticles, and CD polyrotaxanes continue to be developed and modified. In 2011, CDs were called “novel nanoobjects of undeveloped potential” [27]. In 2020, new subclasses of CDs in gene delivery systems have begun to explore this potential and optimize the initial discoveries in the field. Of the literature cited in this work, almost 40% is from the last two years (2019, 2018). The achievements of these groups are complex engineering designs which are more tightly controlled and allow for more specificity of outcome.

Systems incorporating CD allow for modularity in the design. Self-assembly allows for the simple fabrication of complex systems, making delivery of multiple therapeutics facile. Of course, there are still challenges. Guest recognition has been well studied, but delivery from the inner CD pocket can be affected by the larger polymer structure. It will likely not be so easy that any small molecule drug can be switched out for another – differing structures and charges will likely result in differing release profiles. More research needs to be done to fully understand the mechanisms behind these controlled release systems, so that we can better predict which functionalities can be mixed and matched. As we continue to move towards combinatorial and personalized care, it is important that the treatment systems we design make allowances for this direction. CD can fulfill many roles in delivery systems, making it an important addition to platforms that require modularity. Our hope is that researchers in the field of gene delivery will consider the ways that they can add combinatorial therapy to their systems of interest. With the work outlined here, where CD has already been incorporated, it is the authors' belief that the field is already moving in this direction, and this will show drastic improvements when moving to *in vivo* models.

6. CONFLICT OF INTEREST DISCLOSURE

RM Haley, R Gottardi, RS Langer, and MJ Mitchell all declare that they have no conflict of interest.

7. DECLARATION OF INFORMED CONSENT OR ANIMAL STUDIES

No animal or human studies were carried out by the authors for this article.

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